

II. REMARKS

Formal Matters

Claims 15-21 and 66 are pending after entry of the amendments set forth herein.

Claims 15-21 and 66 were examined. Claims 15-17, 19-21, and 66 were rejected. Claim 18 was objected to. Claims 1-14 and 22-29 were withdrawn from consideration.

Claim 15 is amended. The amendments to claim 15 were made solely in the interest of expediting prosecution, and are not to be construed as acquiescence to any objection or rejection of any claim. Support for the amendments to claim 15 is found in the claims as originally filed, and throughout the specification, in particular at the following locations: page 7, lines 24-25. Accordingly, no new matter is added by these amendments.

Claims 1-14 and 22-29 are canceled without prejudice to renewal, without intent to acquiesce to any rejection, and without intent to surrender any subject matter encompassed by the canceled claims. Applicants expressly reserve the right to pursue any canceled subject matter in one or more continuation and/or divisional applications.

Applicants respectfully request reconsideration of the application in view of the remarks made herein.

Examiner Interview

The undersigned Applicants' representative thanks Examiner Hutson for the courtesy of a telephonic interview which took place on November 21, 2005, and which was attended by Examiner Hutson and Applicants' representative Paula A. Borden.

During the interview, the rejection of claims 15-17, 19-21, and 66 under 35 U.S.C. §112, first paragraph, was discussed.

Claim objections

Claims 15 and 18 were objected to. The Office Action stated that claim 15 recites "diacylglycerol-*O*-acyltransferases (DGAT) modulatory activity"; and stated that "acyltransferases" should not be plural.

Claim 15 is amended to recite "diacylglycerol-*O*-acyltransferase (DGAT) modulatory activity."

Rejection under 35 U.S.C. §112, first paragraph

Claims 15-17, 19-21, and 66 were rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement.

The instant specification provides amino acid sequences of at least three DGAT polypeptides.

The specification describes a number of species of DGAT polypeptides. For example, the specification provides the amino acid sequence of a human DGAT polypeptide comprising the amino acid sequence set forth in SEQ ID NO:6; a mouse DGAT polypeptide comprising the amino acid sequence set forth in SEQ ID NO:7; and a plant DGAT polypeptide comprising the amino acid sequence set forth in SEQ ID NO:10. Specification, page 9, lines 8-24. Thus, the specification provides at least three DGAT amino acid sequences.

The instant specification provides ample description of how to determine DGAT activity.

The instant specification teaches how to determine whether an agent modulates DGAT activity. The specification provides a description of how to carry out a screening assay. Specification, page 21, line 18 to page 23, line 9. The specification provides a working example of how to measure DGAT activity. Specification, page 41, lines 10-21.

Given the guidance provided in the specification, combined with the knowledge in the art, those skilled in the art could carry out the claimed screening assay without undue experimentation.

The instant specification discusses structural features of DGAT polypeptides.

Structural features of DGAT polypeptides are discussed in the instant application. As discussed in the specification, there is a serine residue, which is also found in acylCoA:cholesterol acyl transferase (ACAT), that was reported to be important for ACAT activity, and is conserved in DGAT. Specification, page 39, lines 29-31; citing Cao et al. ((1996) *J. Biol. Chem.* 271:14642-14648). This conserved serine residue is depicted in Figure 2A of Cases et al. ((1998) *Proc. Natl. Acad. Sci. USA* 95:13018; a copy of which is provided herewith as Exhibit 1, and which has been previously submitted in an Information Disclosure Statement). The specification also notes that the carboxyl-terminal amino acid sequence is conserved. Specification, page 39, lines 25-27.

The specification further states that a DGAT polypeptide has hydrophobic domains and about 9 transmembrane domains. Specification, page 39, line 31 to page 40, line 5. These regions are indicated by a hydrophobicity plot, as depicted in Figure 2B of Exhibit 1.

Those skilled in the art could readily identify conserved residues and domains.

Those of ordinary skill in the art, given the DGAT amino acid sequences provided in the instant application, and given standard alignment software, could readily align the sequences and identify conserved amino acids or regions. Those of ordinary skill in the art would know that conserved regions are generally less tolerant of change than non-conserved regions. For example, Bouvier-Navé et al. ((2000) *FEBS Lett.* 267:85-96; a copy of which is provided herewith as Exhibit 2) provides an amino acid sequence alignment of an *A. thaliana* DGAT, a *N. tabacum* DGAT, a putative human DGAT (referred to as "HsARGP"), a mouse DGAT, and a *C. elegans* DGAT. Exhibit 2, Figure 1. Exhibit 2 notes the hydrophobic domains, as discussed in the instant application. Exhibit 2, page 89, column 1, first full paragraph. Exhibit 2 further notes the presence of a conserved domain (AELLCFGDREFYKDWW). Exhibit 2, page 89, column 1, first full paragraph. Exhibit 2 notes the conserved serine discussed in the instant application. Exhibit 2, page 89, column 1, first full paragraph. Exhibit 2 further notes the presence of conserved arginine clusters. Exhibit 2, page 89, column 1, first full paragraph. Thus, those of ordinary skill in the art, given the amino acid sequences provided in the instant application, could readily identify conserved domains and residues, using nothing more than straightforward amino acid alignment.

Conclusion as to the rejection under 35 U.S.C. §112, first paragraph

Because the instant specification teaches how to make and use the instant screening methods as claimed, such that those of ordinary skill in the art could readily practice the claimed invention without undue experimentation, instant claims 15-17, 19-21, and 66 meet the enablement requirement of 35 U.S.C. §112, first paragraph.

Applicants submit that the rejection of claims 15-17, 19-21, and 66 under 35 U.S.C. §112, first paragraph, has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

III. CONCLUSION

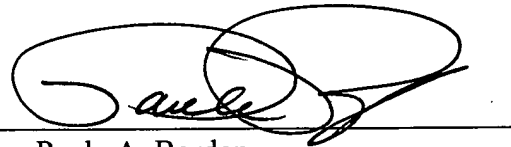
Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number UCAL-105 CIP2.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: Nov. 23, 2005

By: _____



Paula A. Borden
Registration No. 42,344

BOZICEVIC, FIELD & FRANCIS LLP
1900 University Avenue, Suite 200
East Palo Alto, CA 94303
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis

(fatty acid/cloning/expressed sequence tag/glycerolipid)

SYLVAINE CASES^{*†}, STEVEN J. SMITH^{*†}, YAO-WU ZHENG[†], HEATHER M. MYERS^{*}, STEVEN R. LEAR^{‡§}, ERIC SANDE^{*}, SABINE NOVAK^{*†}, COLIN COLLINS[¶], CARRIE B. WELCH^{||**}, ALDONS J. LUSIS^{||}, SANDRA K. ERICKSON^{‡§}, AND ROBERT V. FARESE, JR.^{*§††}

^{*}Gladstone Institute of Cardiovascular Disease, [†]Cardiovascular Research Institute, and [§]Department of Medicine, University of California, San Francisco, CA 94143; [‡]Veterans Administration Medical Center, San Francisco, CA 94121; [§]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720; and [¶]Departments of Microbiology and Molecular Genetics, and Medicine, and Molecular Biology Institute, University of California, Los Angeles, CA 90095

Communicated by Richard J. Havel, University of California, San Francisco, CA, August 24, 1998 (received for review June 24, 1998)

ABSTRACT Triacylglycerols are quantitatively the most important storage form of energy for eukaryotic cells. Acyl CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the terminal and only committed step in triacylglycerol synthesis, by using diacylglycerol and fatty acyl CoA as substrates. DGAT plays a fundamental role in the metabolism of cellular diacylglycerol and is important in higher eukaryotes for physiologic processes involving triacylglycerol metabolism such as intestinal fat absorption, lipoprotein assembly, adipose tissue formation, and lactation. DGAT is an integral membrane protein that has never been purified to homogeneity, nor has its gene been cloned. We identified an expressed sequence tag clone that shared regions of similarity with acyl CoA:cholesterol acyltransferase, an enzyme that also uses fatty acyl CoA as a substrate. Expression of a mouse cDNA for this expressed sequence tag in insect cells resulted in high levels of DGAT activity in cell membranes. No other acyltransferase activity was detected when a variety of substrates, including cholesterol, were used as acyl acceptors. The gene was expressed in all tissues examined; during differentiation of NIH 3T3-L1 cells into adipocytes, its expression increased markedly in parallel with increases in DGAT activity. The identification of this cDNA encoding a DGAT will greatly facilitate studies of cellular glycerolipid metabolism and its regulation.

Acyl CoA:diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) is a microsomal enzyme that plays a central role in the metabolism of cellular glycerolipids (for reviews, see refs. 1 and 2). DGAT catalyzes the only committed step in triacylglycerol synthesis by using diacylglycerol (DAG) and fatty acyl CoAs as its substrates. DAG used in the DGAT reaction can be derived from the hydrolysis of phosphatidic acid produced by the *de novo* synthesis pathway from glycerol-3-phosphate (Fig. 1). Alternatively, DAG can be derived from the esterification of monoacylglycerol (MAG), a pathway of importance in intestinal fat absorption (3), and from the hydrolysis of triacylglycerol or phospholipids. Inasmuch as DAG is a precursor for phospholipid synthesis and is an important signaling molecule that activates protein kinase C (4), DGAT activity potentially could regulate these cellular processes. Because of its role in triacylglycerol synthesis and energy storage, DGAT also may be involved in intestinal fat absorption (3), lipoprotein assembly and the regulation of plasma triacylglycerol concentrations (1, 5), fat storage in adipocytes (6), energy metabolism in muscle (7), milk production (1), and egg

production, including mammalian oocytes (8). In plants, DGAT has an important function in the generation of seed oils (9). DGAT activity also has been found in *Mycobacteria* (10) and *Streptomyces* (11), and in the lipid bodies of fungi (12) and insects (13).

Although it has been partially purified (14, 15), DGAT has been difficult to isolate because it is an intrinsic membrane protein. Through homology searches of the expressed sequence tag (EST) databases by using coding sequences from acyl CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26), an acyltransferase that synthesizes cholesterol esters from cholesterol and fatty acyl CoA substrates (16), we identified an EST clone that shares homology with sequences in the ACAT C terminus. In this study, we demonstrate that this recently identified cDNA does not encode another ACAT, but in fact encodes a DGAT.^{‡†} The expression of a mouse cDNA for this EST in insect cells resulted in high levels of a membrane-associated acyltransferase activity specific for DAG. This gene and its encoded activity then were characterized in detail.

MATERIALS AND METHODS

Cloning of DGAT cDNA. ESTs [accession nos. R07932 (human) and W10786 (mouse)] with sequence similarity to ACAT were identified from BLAST database searches. The 5' end of the DGAT cDNA was obtained by using 5' rapid amplification of cDNA ends (RACE) and a mouse spleen Marathon Ready cDNA library (CLONTECH). Sequences have been deposited in GenBank (accession no. AF078752).

Insect Cell Expression Studies. DGAT coding sequences with or without an N-terminal FLAG epitope (IBI/Kodak, New Haven, CT) (MGDYKDDDDG-, epitope underlined) were subcloned into pVL1392 (PharMingen). High titers of recombinant baculoviruses were obtained by cotransfection of baculovirus transfer vectors with viral BaculoGold DNA (PharMingen), followed by plaque purification and amplification in Sf9 cells [cultured in Grace's medium (Life Technologies, Grand Island, NY) and 10% fetal bovine serum]. H5 insect cells [cultured in serum-free Express-Five medium (Life

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; DAG, diacylglycerol; DGAT, acyl CoA:diacylglycerol acyltransferase; EST, expressed sequence tag; MAG, monoacylglycerol; MOI, multiplicity of infection.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF078752).

^{**}Present address: Columbia University, College of Physicians and Surgeons, New York, NY, 10032

^{††}To whom reprint requests should be addressed at: Gladstone Institute of Cardiovascular Disease, P.O. Box 419100, San Francisco, CA 94141-9100. e-mail: bfarese@gladstone.ucsf.edu.

^{‡†}Portions of this work were presented at the Experimental Biology meeting (San Francisco, 1998) and have been published in abstract form (34).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/9513018-6\$2.00/0 PNAS is available online at www.pnas.org.

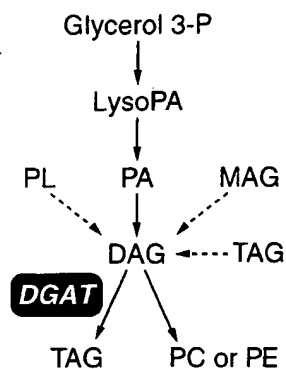


Fig. 1. Role of DGAT in glycerolipid metabolism. DAG used by DGAT potentially originates from hydrolysis of phosphatidic acid (PA), from the esterification of 2-monoacylglycerol (MAG), or from triacylglycerol (TAG) or phospholipid (PL) hydrolysis. The MAG pathway is thought to be especially important in enterocytes of the small intestine (3). P, phosphate; LysoPA, lysophosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Technologies)] were plated on day 0 (8.5×10^6 cells/100-mm dish) and infected on day 1 with high titers of virus at a multiplicity of infection (MOI) that was empirically determined. On day 3, cells were collected by centrifugation and washed twice with PBS. Cell pellets were homogenized by 10 passages through a 27-gauge needle in 0.1 M sucrose, 50 mM KCl, 40 mM KH_2PO_4 , and 30 mM EDTA (pH 7.2). Total membrane fractions ($100,000 \times g$ pellet) were resuspended in the homogenization buffer and frozen (-80°C). Immunoblots of membrane proteins (75 μg) were performed with the anti-FLAG M2 mAb (IBI/Kodak).

For metabolic labeling, H5 insect cells were plated on day 0 (2.9×10^6 cells/60-mm dish) and infected on day 1 with high titers of viruses. On day 3, cells were washed and incubated in methionine- and cysteine-free medium (SF900 II, Life Technologies) for 2 h, followed by incubation in the same medium containing 715 μCi of [^{35}S]methionine and [^{35}S]cysteine (Pro-Mix; Amersham; 1 Ci = 37 GBq) for 1 h. Cells were washed twice with PBS and collected by low-speed centrifugation. The cell pellet was resuspended in 0.5 ml of 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 (pH 7.4) and sonicated. Cellular proteins (100 μg) were analyzed by SDS/PAGE and autoradiography.

For ACAT assays, cell-membrane proteins (100 μg) were assayed by using [^{14}C]oleoyl CoA (51 mCi/mmol, Amersham) and cholesterol/egg phosphatidylcholine (PC) liposomes (molar ratio = 0.7) as described (17). In some assays, other acyl acceptors were substituted for cholesterol in the liposomes at a molar ratio of 0.2 (acceptor/egg PC). Incorporation of the [^{14}C]oleoyl group into products was assessed by TLC, followed by autoradiography. DGAT assays were based on assays optimized for rat liver (15, 18) (S.K.E., K. Pella, and S.R.L., unpublished data). The incorporation of [^{14}C]oleoyl CoA into triacylglycerol was measured under apparent V_{MAX} conditions by using exogenous DAG provided as DAG/egg PC liposomes (molar ratio ≈ 0.16). Cell-membrane proteins (20–25 μg) were assayed in 0.25 M sucrose, 1 mM EDTA, 150 mM MgCl_2 , and 100 mM Tris-HCl (pH 7.5) containing 250 μg of BSA and 20 μg of DAG in liposomes and 5 nmol [^{14}C]oleoyl CoA (40,000 dpm/nmol) (final volume, 0.2 ml). Reactions were carried out for 5 min, and the products were analyzed as described (19). Similar assays were performed with 1-stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycerol (53 mCi/mmol, Amersham) diluted to a final activity of 38,000 dpm/nmol with unlabeled 1,2-diacyl-*sn*-glycerol and unlabeled oleoyl CoA.

Relative triacylglycerol and DAG masses were determined by total lipid extraction of membranes or cells followed by TLC, iodine vapor visualization, photography of the plates,

and densitometric analysis. Triolein standards were used to estimate the mass of triacylglycerols, and DAG units were estimated relative to one another. Triacylglycerol values were normalized to 1 for wild-type virus-infected cell membranes to correct for inter-experiment variability.

mRNA Expression. Human Multiple Tissue Northern blots (CLONTECH) were hybridized with a ^{32}P -labeled 1.1-kb human DGAT fragment from the human EST. For mouse tissues, total RNA was prepared with Trizol reagent (Life Technologies), and samples (10 μg) were analyzed by Northern blot with a ^{32}P -labeled, 1-kb mouse DGAT fragment from the mouse EST. Blots were stripped and sequentially reprobed for glyceraldehyde-3-phosphate dehydrogenase and 28S RNA (20). Bands in autoradiograms from the 3T3-L1 experiments were quantified with a PhosphorImager (Fuji Medical Systems, Stamford, CT).

NIH 3T3-L1 Differentiation. NIH 3T3-L1 fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM L-glutamine. 3T3-L1 cell differentiation into adipocytes was induced by incubating confluent monolayers of cells in serum-containing medium supplemented with 10^{-5} M dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 $\mu\text{g}/\text{ml}$ insulin (21).

Gene Mapping. Primers derived from the human EST sequences were used to identify genomic clones in an arrayed bacteria artificial chromosome (BAC) library according to the manufacturer's protocol (Research Genetics, Huntsville, AL). The BAC clone was mapped to chromosome 8qter by fluorescent *in situ* hybridization (22). The clone (RMC08P051) may be requested from the website <http://rmc-www.lbl.gov>. Linkage analysis for mouse gene mapping was performed with a panel of 67 progeny derived from an interspecific backcross [(C57BL/6J \times *Mus spretus*)F₁ \times C57BL/6J] (23). This backcross panel has been typed for more than 400 loci throughout the genome (24). Briefly, parental strain DNAs were screened for restriction fragment-length variants by restriction enzyme digestion and hybridization with a radiolabeled, 1-kb mouse DGAT cDNA fragment as described (23). Filters were washed in $1.0\times$ SSC/0.1% SDS at 50°C for 20 min. Autoradiograms were exposed for 3 days at -70°C . Linkage to previously typed chromosomal markers was detected by using MAP MANAGER version 2.6.5, and loci were ordered by minimizing the number of recombination events between DGAT and the markers (25).

RESULTS

Through homology searches of the EST databases using coding sequences from ACAT, we identified an EST clone that shared homology with sequences in the ACAT C terminus. The translation of a full-length cDNA for this EST predicts an ORF encoding a 498-aa protein that is $\approx 20\%$ identical to mouse ACAT (Fig. 24), with the most highly conserved regions in the C terminus. The predicted protein sequence contains a potential N-linked glycosylation site and a putative tyrosine phosphorylation site. A serine residue found in ACAT that is necessary for enzyme activity (26) appears to be conserved. The protein has multiple hydrophobic domains and 6–12 possible transmembrane domains (Fig. 2B). Analysis by a transmembrane region prediction program (http://ulrec3.unil.ch/software/TMPRED_form.html) favors nine transmembrane domains (amino acids 96–114, 140–157, 174–198, 200–218, 293–311, 337–360, 412–434, 436–456, and 461–484).

Given the 20% sequence identity to ACAT, experiments were designed to test whether this cDNA encoded an enzyme that catalyzed cholesterol esterification. FLAG epitope tagged (at the N terminus) or untagged versions of the cDNA were expressed in H5 insect cells by using a baculovirus expression system. Cells infected with the virus containing this cDNA

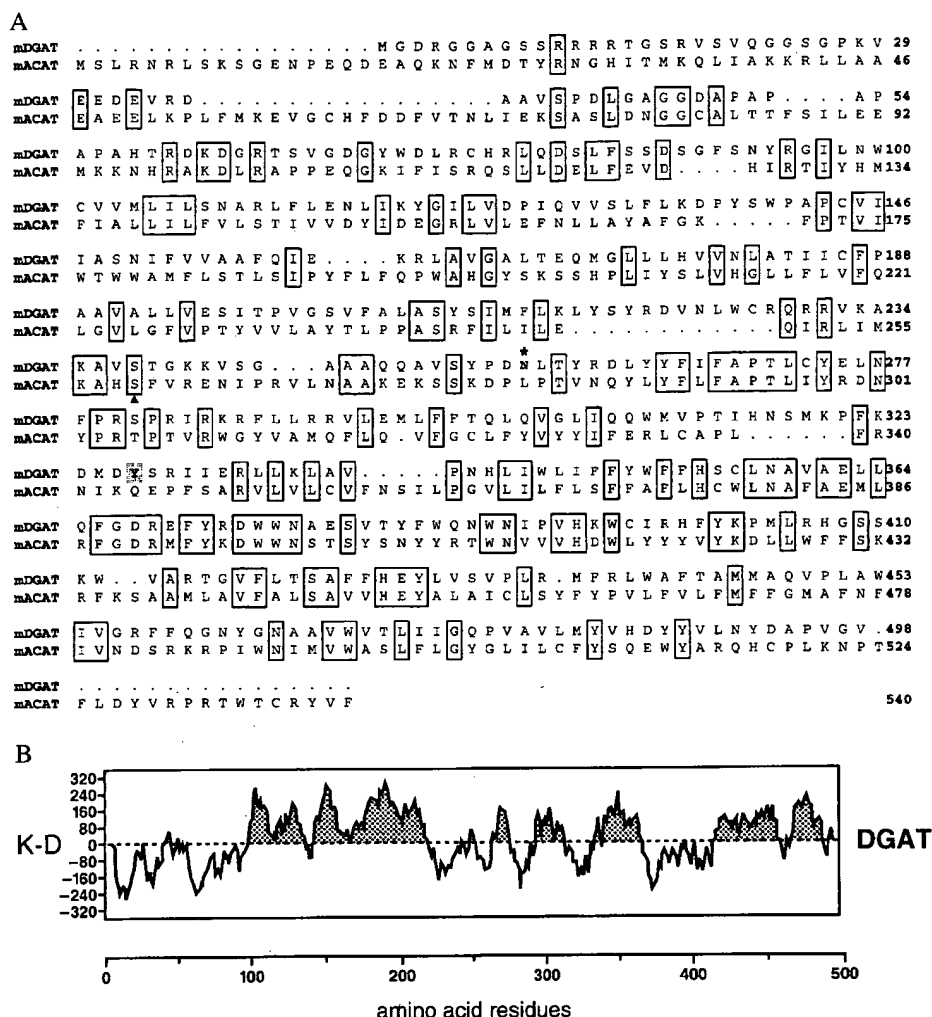


Fig. 2. The mouse DGAT protein. (A) Predicted amino acid sequence of the mouse DGAT cDNA. The predicted amino acid sequence of mouse DGAT (mDGAT) is shown in alignment with mouse ACAT (mACAT) (32). The two sequences are $\approx 20\%$ identical (identical residues are boxed). A potential N-linked glycosylation site (asterisk) and tyrosine phosphorylation site (shaded). A serine residue in ACAT known to be necessary for catalytic function is also indicated (triangle). (B) Hydrophobicity plot of DGAT as assessed by Kyte–Doolittle (K–D) analysis (33). Hydrophobic regions are shaded.

expressed an ≈ 47 -kDa protein at high levels in the membrane fraction (Fig. 3A) but lacked detectable cholesterol esterification activity as compared with ACAT virus-infected cells (Fig. 3B). However, further analysis of TLC plates from these assays revealed that membranes from these cells had significantly increased triacylglycerol mass (as assessed by I_2 visualization) (data not shown) and incorporated significantly more [14 C]-oleoyl CoA into triacylglycerols than did membranes from wild-type virus-infected cells (197 vs. 55 pmol/mg protein per min). These data suggested that the identified cDNA encoded a DGAT.

DGAT activity was measured in membranes from H5 insect cells expressing the putative DGAT cDNA and found to be more than 5-fold higher than in membranes from wild-type virus-infected cells (Fig. 3B). The DGAT activity level increased proportionately with the amount of FLAG-tagged protein expressed in membranes isolated from cells harvested at different time points after infection (Fig. 3C). DGAT activity levels in membranes from cells expressing the cDNA were similar regardless of whether [14 C]DAG or [14 C]oleoyl CoA was used as the labeled substrate (Fig. 3D). In the absence of added oleoyl CoA, [14 C]DAG was not incorporated into triacylglycerols. In addition, [3 H]oleic acid was not incorporated into triacylglycerols in membranes from DGAT virus-

infected cells (7 ± 6 vs. 49 ± 47 pmol triacylglycerol/mg protein per min for wild type, $n = 3$), establishing the requirement for a fatty acyl CoA. Triacylglycerol mass was increased more than 10-fold in membranes from DGAT virus-infected cells compared with membranes from wild-type virus-infected cells (11 ± 7 vs. 1 ± 0.5 pg/ μ g membrane protein, $P = 0.04$, $n = 5$). No change in relative DAG mass was observed (0.33 ± 0.05 vs. 0.34 ± 0.12 units for DGAT and wild type, respectively). We also tested a variety of other possible acyl acceptors, including 25-hydroxy-, 26-hydroxy-, 7 α -hydroxy- or 7 β -hydroxycholesterols, 7-ketocholesterol, vitamins D2 and D3, ethanol, β -sitosterol, lanosterol, and ergosterol (shown in Fig. 3E), and vitamin E, retinol, and dehydroepiandrosterone (data not shown), as substrates for the expressed DGAT enzyme. Although [14 C]oleoyl CoA was consistently incorporated into triacylglycerols (by using the endogenous diacylglycerol as the acyl acceptor), it was not incorporated into esters for any other substrate tested, as assessed by autoradiography of TLC plates used to analyze reaction products (Fig. 3E).

DGAT mRNA expression was examined in a cultured cell model of adipocyte differentiation and in mammalian tissues. The mRNA increased markedly (≈ 8 -fold) in parallel with DGAT activity in NIH 3T3-L1 cells during their differentiation

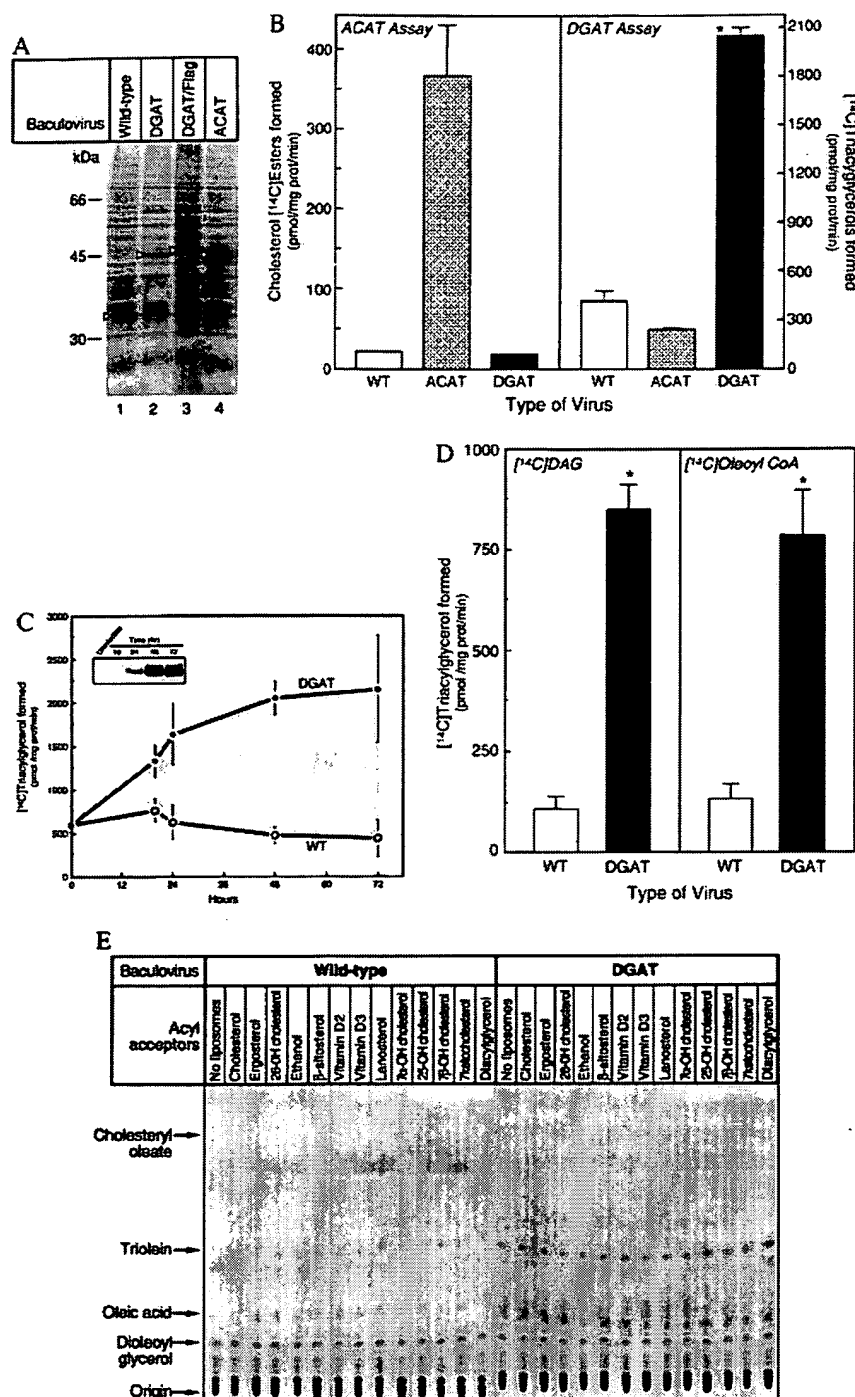


FIG. 3. Enzymatic activities in insect cell membranes expressing DGAT. Cells were infected with wild-type baculovirus (WT), mouse ACAT, or mouse DGAT recombinant baculoviruses, and membranes were assayed for enzymatic activity. (A) Metabolic labeling. Cell proteins, 48 h after infection, were metabolically labeled with [35 S]methionine and [35 S]cysteine, and whole-cell lysates were analyzed by SDS/PAGE and autoradiography. The expression of viral polyhedrin protein (lane 1), mouse DGAT (lane 2), FLAG-tagged mouse DGAT (lane 3), and mouse ACAT (lane 4) is indicated (triangles). (B) ACAT and DGAT activities. Data represent the mean (\pm SE) of five experiments. *, $P < 0.001$, vs. WT. (C) Time course of DGAT virus infection. Insect cell membranes were isolated at the indicated times after infection. Expression of the FLAG-tagged DGAT was detected by immunoblotting with an anti-FLAG antibody (inset), and DGAT activity was measured. The doublet band observed in this experiment was not routinely observed, and its significance is unknown. Data represent the mean (\pm SE) of three experiments. (D) Comparison of the rate of triacylglycerol synthesis with either DAG or oleoyl CoA as the radiolabeled substrate. Assays contained the same amounts of oleoyl CoA (5 nmol) and DAG (2.5 μ g) in all cases. The specific activity for DGAT virus-infected cells is less than that observed in A because of the reduction in DAG substrate concentration (i.e., this experiment was not performed at apparent V_{MAX}). Data are the mean (\pm SE) of five experiments. *, $P < 0.001$ vs. WT. (E) Acyl acceptor specificity of DGAT. Reaction products from wild-type or DGAT virus-infected membranes assayed with [14 C]oleoyl CoA and various acyl acceptor substrates were analyzed by TLC. Note that [14 C]oleoyl CoA is incorporated specifically into triacylglycerols for all reactions containing membranes expressing DGAT. Hydrolysis of the labeled oleoyl CoA to oleic acid (as shown in this experiment) was observed in some, but not all, preparation of membranes expressing DGAT; this finding was associated with membranes expressing the highest levels of DGAT activity.

into adipocytes (Fig. 4*A* and *B*). DGAT mRNA was expressed in every human (Fig. 4*C*) and mouse (data not shown) tissue examined, with the highest expression levels in the small intestine. In addition, expression was detected in mouse adipose tissue (Fig. 4*D*). In the human Northern blots, additional hybridization signals were observed at ≈ 2.2 kb and ≈ 4.0 kb. The significance of these bands is currently unknown.

The human DGAT gene was mapped to human chromosome 8qter by fluorescent *in situ* hybridization. By using an interspecific cross, we mapped the mouse homolog for the DGAT gene (*Dgat*) to a region of chromosome 15 that exhibits homology with human chromosome 8 (Fig. 5). This region of mouse chromosome 15 has exhibited linkage with levels of plasma triacylglycerol-rich lipoproteins in several genetic crosses (24, 27, 28). In each case, the strongest linkage was observed with levels of plasma very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol, but there was also evidence of linkage with levels of plasma triacylglycerols. For example, in a cross between strains MRL and BALB/c, marker D15Mit17 exhibited a lod score of 6.7 for VLDL and LDL cholesterol and 2.7 for triacylglycerols (24).

DISCUSSION

DGAT is a microsomal enzyme that plays a central role in the biosynthesis of cellular triacylglycerols. DGAT has never been

purified to homogeneity, nor has its gene been cloned. In this study, we identified a cDNA encoding a protein that possesses DGAT activity. The identification of this cDNA provides a molecular probe for studying the role of DGAT in biology.

Several findings support the hypothesis that the cDNA we identified encodes a DGAT. First, DGAT activity was more than 5-fold higher in membranes expressing the cDNA than in membranes from wild-type virus-infected cells. Although insect cells, like all eukaryotic cells, synthesize triacylglycerols (2), the ability to express large amounts of the DGAT protein enabled us to detect DGAT-specific activity at high levels, similar to those found in mammalian tissues (14) and considerably above background levels in insect cells. The acyltransferase activity depended on the presence of a fatty acyl CoA substrate and was specific for DAG; there was no activity with cholesterol or a variety of other acyl acceptor substrates. Second, its mRNA expression increased markedly in parallel with DGAT activity in NIH 3T3-L1 cells during their differentiation into adipocytes, a process known to be associated with increases in DGAT activity (29) and triacylglycerol mass accumulation (30). Third, mRNA expression was detected in every mammalian tissue examined, as expected because of the central role of DGAT in cellular glycerolipid metabolism. The highest expression levels were found in the small intestine, consistent with a proposed role for DGAT in intestinal fat

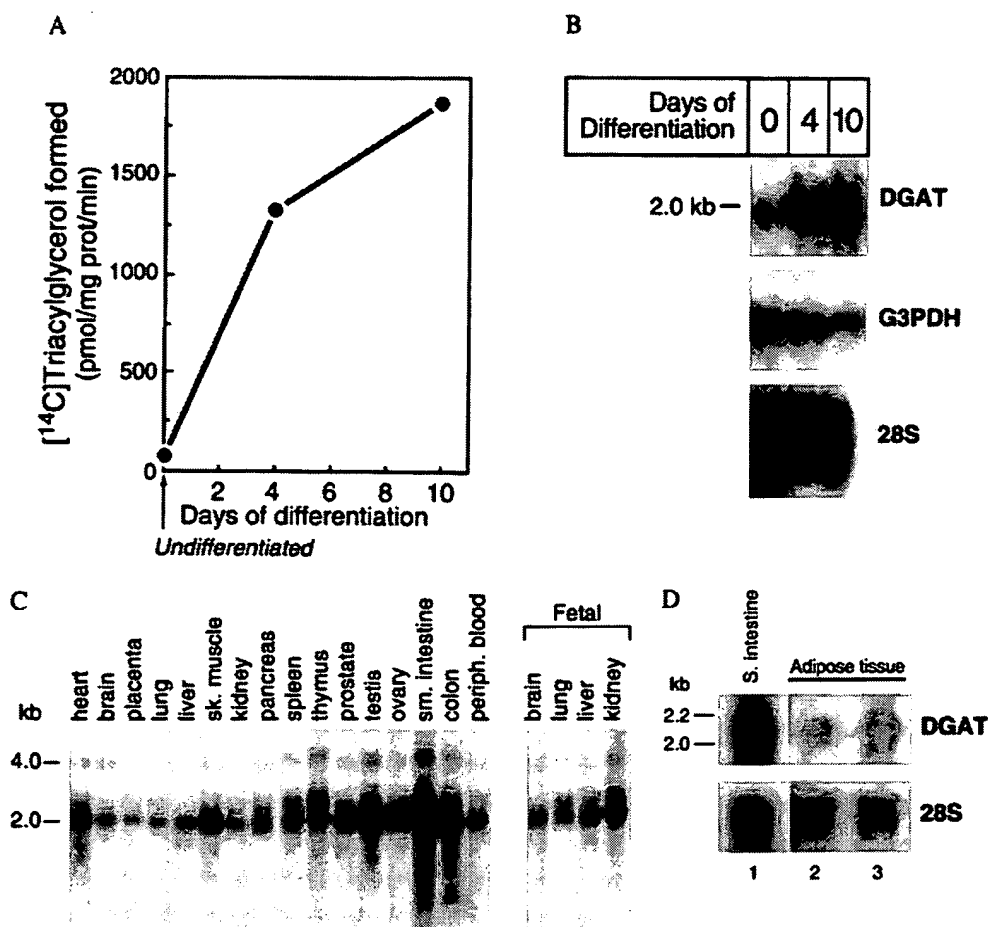


FIG. 4. Analysis of DGAT mRNA expression. (*A*) DGAT activity and (*B*) DGAT mRNA expression during differentiation of 3T3-L1 cells into adipocytes. Mouse 3T3-L1 adipocyte differentiation was induced, and RNA and membranes were isolated from undifferentiated cells or cells harvested 4 and 10 days later. Results are shown for DGAT and two controls for RNA loading [glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and 28S RNA]. Quantitation of DGAT mRNA in triplicate samples, by PhosphorImager analysis and correction for loading relative to 28S RNA (20) as an internal standard, demonstrated that DGAT levels were increased 5-fold by day 4 and 8-fold by day 10 of differentiation. The experiment was repeated three times with similar results. (*C*) DGAT expression in human tissues as assessed by Northern blot analysis. (*D*) DGAT expression in mouse small intestine and adipose tissue from two mice (lanes 2 and 3). The membrane was stripped and reprobed for 28S RNA (20).

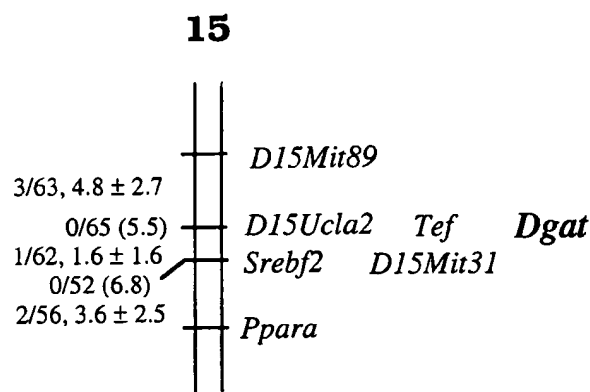


FIG. 5. Mouse chromosomal location of DGAT gene. Genetic mapping of the *Dgat* to mouse chromosome 15 was performed by linkage analysis performed with a panel of 67 progeny from an interspecific backcross [(C57BL/6J \times *Mus spretus*)F₁ \times C57BL/6J] (23). A segment of the chromosome is drawn with the centromere toward the top. The ratios of the number of recombinants to the total number of informative mice and the recombination frequencies \pm SE (in cM) for each pair of loci are indicated. For pairs of loci that cosegregate, the upper 95% confidence interval is shown in parentheses. No recombination was observed between *Dgat*, *D15Ucla2*, and *Tef* (0/65 mice). The DGAT gene has been assigned the name *Dgat*. The data have been deposited in the Mouse Genome Database under accession number MGD-J:44983.

absorption (3, 31). In addition, the mRNA was expressed in adipose tissue, which is known to have a high level of DGAT activity (6). Interestingly, mRNA expression was relatively low in the livers of humans (Fig. 4C) and mice (data not shown), despite the fact that significant DGAT activity is present in the liver (14). The significance of this finding is currently unknown. One possibility is that, although the expression levels are low, the mRNA encodes a very stable protein; alternatively, it is possible that the liver expresses a second DGAT. As a final piece of evidence confirming the identity of this cDNA, we have disrupted the mouse DGAT gene in embryonic stem cells and achieved germ-line transmission of this mutation. Preliminary results indicate that DGAT activity in membranes from embryonic fibroblasts homozygous for the knockout mutation is reduced to \approx 5% or less than that in wild-type fibroblast membranes (S.J.S., S.C., and R.V.F., unpublished observations).

The identification of a DGAT cDNA has significant implications for understanding the regulation of the triacylglycerol biosynthetic pathway and intracellular lipid metabolism. DGAT molecular probes will facilitate *in vivo* studies of the role(s) of DGAT and its regulation in a number of physiologic processes, such as intestinal fat absorption, lipoprotein synthesis and secretion, lactation, and adipose tissue formation. Whether DGAT regulation participates in modulating levels of cellular DAG involved in signaling also can be assessed. Ultimately, understanding DGAT at a molecular level may uncover potential approaches for treating hypertriglyceridemia or obesity in humans.

We thank Drs. W.-L. Kuo and J. Cochran for assistance in mapping the human gene and Dr. J. Borén for help with metabolic labeling experiments. We also thank A. Chen and M. Chow for manuscript preparation, J. Carroll for graphics, S. Ordway and G. Howard for editorial assistance, and Dr. B. Conklin for helpful comments on the manuscript. This work was supported by the J. David Gladstone

Institutes; National Institutes of Health Grants HL57170 (R.V.F.), HL52069 (S.K.E.), and HL42488 (A.J.L.); an American Heart Association Postdoctoral Fellowship Award (S.C.); and a Merit Award from the Department of Veterans Affairs (S.K.E.).

- Bell, R. M. & Coleman, R. A. (1980) *Annu. Rev. Biochem.* **49**, 459–487.
- Lehner, R. & Kuksis, A. (1996) *Prog. Lipid Res.* **35**, 169–201.
- Brindley, D. N. (1991) in *Biochemistry of Lipids, Lipoproteins and Membranes*, eds. Vance, D. E. & Vance, J. E. (Elsevier, Amsterdam), pp. 171–203.
- Nishizuka, Y. (1992) *Science* **258**, 607–614.
- Haagsman, H. P. & Van Golde, L. M. G. (1981) *Arch. Biochem. Biophys.* **208**, 395–402.
- Coleman, R. & Bell, R. M. (1976) *J. Biol. Chem.* **251**, 4537–4543.
- Swanton, E. M. S. & Saggerson, E. D. (1997) *Biochim. Biophys. Acta* **1346**, 93–102.
- Homa, S. T., Racowsky, C. & McGaughey, R. W. (1986) *J. Reprod. Fertil.* **77**, 425–434.
- Lassner, M. (1997) *Lipid Technol.* **9**, 5–9.
- Akao, T. & Kusaka, T. (1976) *J. Biochem. (Tokyo)* **80**, 723–728.
- Olukoshi, E. R. & Packter, N. M. (1994) *Microbiology* **140**, 931–943.
- Kamisaka, Y., Mishra, S. & Nakahara, T. (1997) *J. Biochem. (Tokyo)* **121**, 1107–1114.
- Arrese, E. L., Rojas-Rivas, B. I. & Wells, M. A. (1996) *Arch. Insect Biochem. Physiol.* **31**, 325–335.
- Polokoff, M. A. & Bell, R. M. (1980) *Biochim. Biophys. Acta* **618**, 129–142.
- Andersson, M., Wettsten, M., Borén, J., Magnusson, A., Sjöberg, A., Rustaeus, S. & Olofsson, S.-O. (1994) *J. Lipid Res.* **35**, 535–545.
- Chang, T. Y., Chang, C. C. Y. & Cheng, D. (1997) *Annu. Rev. Biochem.* **66**, 613–638.
- Smith, J. L., Lear, S. R. & Erickson, S. K. (1995) *J. Lipid Res.* **36**, 641–652.
- Ozasa, S., Kempner, E. S. & Erickson, S. K. (1989) *J. Lipid Res.* **30**, 1759–1762.
- Erickson, S. K., Shrewsbury, M. A., Brooks, C. & Meyer, D. J. (1980) *J. Lipid Res.* **21**, 930–941.
- Barbu, V. & Dautry, F. (1989) *Nucleic Acids Res.* **17**, 7115.
- Brasaemle, D. L., Barber, T., Wolins, N. E., Serrero, G., Blanchette-Mackie, E. J. & Londos, C. (1997) *J. Lipid Res.* **38**, 2249–2263.
- Stokke, T., Collins, C., Kuo, W.-L., Kowbel, D., Shadravan, F., Tanner, M., Kallioniemi, A., Kallioniemi, O.-P., Pinkel, D., Deaven, L. & Gray, J. W. (1995) *Genomics* **26**, 134–137.
- Warden, C. H., Mehrabian, M., He, K.-Y., Yoon, M.-Y., Diep, A., Xia, Y.-R., Wen, P.-Z., Svenson, K. L., Sparkes, R. S. & Lusis, A. J. (1993) *Genomics* **18**, 295–307.
- Welch, C. L., Xia, Y.-R., Shechter, I., Farese, R., Mehrabian, M., Mehdizadeh, S., Warden, C. H. & Lusis, A. J. (1996) *J. Lipid Res.* **37**, 1406–1421.
- Manly, K. F. (1993) *Mamm. Genome* **4**, 303–313.
- Cao, G., Goldstein, J. L. & Brown, M. S. (1996) *J. Biol. Chem.* **271**, 14642–14648.
- Purcell-Huynh, D. A., Weinreb, A., Castellani, L. W., Mehrabian, M., Doolittle, M. H. & Lusis, A. J. (1995) *J. Clin. Invest.* **96**, 1845–1858.
- Gu, L., Johnson, M. W. & Lusis, A. J. *Arterioscler. Thromb. Vasc. Biol.*, in press.
- Coleman, R. A., Reed, B. C., Mackall, J. C., Student, A. K., Lane, M. D. & Bell, R. M. (1978) *J. Biol. Chem.* **253**, 7256–7261.
- Green, H. & Kehinde, O. (1975) *Cell* **5**, 19–27.
- Mansbach, C. M., II (1973) *Biochim. Biophys. Acta* **296**, 386–402.
- Uelmen, P. J., Oka, K., Sullivan, M., Chang, C. C. Y., Chang, T. Y. & Chan, L. (1995) *J. Biol. Chem.* **270**, 26192–26201.
- Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
- Cases, S., Smith, S. J., Zheng, Y.-W., Myers, H. M., Sande, E. R., Novak, S., Lear, S. R., Erickson, S. K. & Farese, R. V., Jr. (1998) *FASEB J.* **12**, A814.